EXHIBIT D

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REVIEW

The Double-Stranded RNA-Dependent Protein Kinase PKR: Structure and Function

MICHAEL J. CLEMENS and ANDROULLA ELIA

ABSTRACT

This review describes the structure and function of the interferon (IFN)-inducible, double-stranded RNA-activated protein kinase PKR. This protein kinase has been studied extensively in recent years, and a large body of evidence has accumulated concerning its expression, interaction with regulatory RNA and protein molecules, and modes of activation and inhibition. PKR has been shown to play a variety of important roles in the regulation of translation, transcription, and signal transduction pathways through its ability to phosphorylate protein synthesis initiation factor eIF2, I-κB (the inhibitor of NF-κB), and other substrates. Expression studies involving both the wild-type protein and dominant negative mutants of PKR have established roles for the enzyme in the antiviral effects of IFNs, in the responses of uninfected cells to physiologic stresses, and in cell growth regulation. The possibility that PKR may function as a tumor suppressor and inducer of apoptosis suggests that this IFN-regulated protein kinase may be of central importance to the control of cell proliferation and transformation.

INTRODUCTION

It is now over 20 years since the discovery of a protein kinase that was shown to mediate the inhibition of protein synthesis when double-stranded RNA (dsRNA) was added to the reticulocyte lysate cell-free translation system.[1–5] Interest in this enzyme increased dramatically when it was demonstrated that it was not only a peculiarity of immature red cells but was also inducible in a wide range of cell types by treatment with interferons (IFNs)[6–9] and was likely to be responsible for at least part of the antiviral and antiproliferative effects of these cytokines.[10–13]

The name of the dsRNA-dependent protein kinase has now been standardized as PKR (protein kinase RNA regulated),[14] but, previously, this enzyme has also been widely known as DAI, dsI, and p68, as well as having a number of other names. PKR belongs to a small family of enzymes that have a common substrate, the smallest (α) subunit of polypeptide chain initiation factor eIF-2.[15–17] Homologs of PKR have been identified not only in human, mouse, rat, and rabbit cells but even in plants.[18,19] More distant relatives of PKR, which retain the same substrate specificity, include the protein kinases GCN2 (involved in the general control of amino acid biosynthesis in the yeast *Saccharomyces cerevisae*) and the heme-regulated kinase HCR or HRI (involved in the regulation of reticulocyte protein synthesis). Both of these enzymes are, however, activated in completely different ways from PKR and are not considered further in this review.

The phosphorylation of eIF-2α has proved to be a widely used mechanism for the control of translation (reviewed in references 16 and 20). In addition to having effects on this pathway, PKR has been implicated in the regulation of transcription and signal transduction and thus impinges on a wide range of biologic activities. For these reasons, PKR has become the most widely studied of the eIF-2α kinases and has been the subject of a number of earlier reviews.[13,15,20–27]

STRUCTURE OF THE *PKR* GENE AND PROTEIN

Genomic sequences have been described for the mouse and human *PKR* genes.[28–31] The mouse gene consists of 16 exons and 15 introns, whereas the human equivalent has one more exon and intron than that of the mouse.[30,31] There is considerable conservation of exon and intron structure between the two species, although the 5′ untranslated region of the human mRNA is substantially longer than its equivalent in the mouse PKR mRNA. The *PKR* gene is present on mouse chromosome 17 E2[32] and is transcribed from a promoter containing a large

Division of Biochemistry, Department of Cellular and Molecular Sciences, St. George's Hospital Medical School, London, U.K.

number of potential regulatory elements, including interferon (IFN)-sensitive ISRE and GAS elements, the interleukin-6 (IL-6)-sensitive APRF sequence, and NF-κB and NF-IL-6 elements.[28] The human *PKR* gene is located on chromosome 2p21-p22[32,33] and has a similar level of complexity in its promoter.[34]

The sequences of cDNAs for human, mouse, and rat PKR have all been characterized.[35–37] The deduced structure of the protein reveals a number of discrete modules (Fig. 1). The 11 structural motifs characteristic of protein kinases in general are found in the C-terminal half of the protein, whereas the N-terminal half contains two dsRNA-binding motifs that are necessary for the interaction of the enzyme with its activators. These are considered in more detail subsequently.

Structure of the RNA binding domains of PKR

The N-terminal half of PKR has been demonstrated to be essential for dsRNA binding,[38,39] and mutational analysis has identified two regions that contain dsRNA-binding motifs with a number of sequence similarities (Fig. 1 and Table 1). These two motifs, designated dsRBM1 and dsRBM2 (or R_I and R_{II}), are located at positions 11-77 and 101-167, respectively, in the amino acid sequence of the human protein.[40–44] Studies on deletion and substitution mutants of these regions have suggested that dsRBM1 is both necessary and sufficient for dsRNA binding.[39,45] Mutation of an invariant lysine at residue 64 in dsRBM1 strongly inhibited or abolished RNA binding ability.[39,46,47] Further mutational analyses of the dsRNA-binding domains in PKR have identified several other residues as being absolutely required for ligand binding[46,47] (Table 2). A peptide corresponding to amino acids 60-80 could block activation of PKR by dsRNA.[48] Although some studies suggest that dsRBM2 may not be essential for dsRNA binding to PKR, other reports have implicated this region as having a role in optimizing the binding of dsRNA to dsRBM1[41] or even being of equal importance to dsRBM1.[47]

The dsRBMs are similar to sequences found in many other dsRNA-binding proteins[49] (Table 1). The latter include a number of viral gene products,[50–53] mammalian and amphibian proteins of diverse functions,[41,49,54,55] and the product of the *Drosophila* gene *Staufen*.[56,57] Such motifs appear to be of widespread occurrence throughout the living kingdom and are presumably of ancient evolutionary origin, as the dsRNA-specific ribonuclease of *Escherichia coli*, RNase III, also contains a similar sequence.[58] NMR techniques have been used to demonstrate that the *Staufen* dsRNA-binding domain has an α-β-β-β-α structure, in which two α-helices lie on one face of a three-stranded antiparallel β-sheet.[56] A similar structure has been elucidated for the RNase III domain.[58]

Although relatively little is known of how dsRNA associates with the two dsRNA-binding motifs found in PKR, the region containing these motifs is again predicted to be α-helical.[41,42] It has been suggested that the N-terminal and dsRBM2 regions flanking dsRBM1 may stabilize the α-helical region of the motif or may correctly orientate this region to facilitate interaction with RNA (reviewed in reference 39). Initial studies on this subject have shown that both dsRBM1 and dsRBM2 form α-helices that are capable of binding to dsRNA.[41] Within these regions there is a high proportion of lysine and arginine residues. However, it is probable that binding is not a straightforward case of ionic attraction between these basic amino acids and the negative charges on the dsRNA backbone, as other structural features of the dsRBMs are important for binding. Unlike the binding to DNA of proteins with motifs, such as the zinc finger, helix-turn-helix, or leucine zipper, which all facilitate DNA-protein interactions by inserting the α-helix of the protein into the major groove of the DNA, PKR (and the other

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1MAGDLSAGFF MEELNTYRQK QGVVLKYQEL PNSGPPHDRR FTFQVIIDGR EFPEGEGRSK KEAKNAAAKL

71AVEILNKEKK AVSPLLLTTT NSSEGLSMGN YIGLINRIAQ KKRLTVNYEQ CASGVHGPEG FHYKCKMGQK

141EYSIGTGSTK QEAKQLAAKL AYLQILSEET SVKSDYLSSG SFATTCESQS NSLVTSTLAS ESSSEGDFSA

211DTSEINSNSD SLNSSSLLMN GLRNNQRKAK RSLAPRFDLP DMKETKYTVD KRFGMDFKEI ELIGSGGFGQ

281VFKAKHRIDG KTYVIKRVKY NNEKAEREVK ALAKLDHVNI VHYNGCWDGF DYDPETSDDS LESSDYDPEN

351SKNSSRSKTK CLFIQMEFCD KGTLEQWIEK RRGEKLDKVL ALELFEQITK GVDYIHSKKL IHRDLKPSNI

421FLVDTKQVKI GDFGLVTSLK NDGKRTRSKG TLRYMSPEQI SSQDYGKEVD LYALGLILAE LLHVCDTAFE

491TSKFFTDLRD GIISDIFDKK EKTLLQKLLS KKPEDRPNTS EILRTLTVWK KSPEKNERHT C
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FIG. 1. Sequence and domain structure of human PKR. The amino acid sequence is derived from the nucleotide sequence of the cDNA for human PKR[35] (EMBL accession number M35663). The following functional domains are boxed. Amino acids 11-77: dsRNA-binding motif 1; amino acids 101-167: dsRNA-binding motif 2 (see Table 1); amino acids 233-271: basic domain essential for enzymatic activity[92]; amino acids 273-281 and 296: ATP binding/phosphotransferase sites; amino acids 366-415: substrate recognition site. The protein kinase subdomains I–XI are located between amino acids 273 and 526, with the putative active site at residues 410-422. Single amino acids or short sequences essential for dsRNA binding, protein dimerization (see Table 1), and kinase activity[64] are shown as bold, single-underlined and double-underlined residues, respectively. Not marked on the sequence are the region believed to be involved in binding of the PKR inhibitor p58 (residues 244-296)[65] and a potential PEST region (residues 323-351) that may target the protein for proteolytic cleavage.

TABLE 1. dsRBM MOTIFS IN DOUBLE-STRANDED RNA BINDING PROTEINS[a]

Protein	*Amino acid residues*	*Sequence*
Hu-PKR (dsRBM1)	11-77	MEELNTYRQKQGVVLKYQELPNSGPPHDRRFTFQVIIDGREFPEGEGRSKKEAKNAAAKLAVEILNK
Hu-PKR[b] (dsRBM2)	101-167	YIGLINRIAQKKRLTVNYEQCASGVHGPEGFHYKCKMGQKEYSIGTGSTKQEAKQLAAKLAYLQILS
Mu-PKR[b] (dsRBM1)	10-76	MDKLNKYRQMHGVAITYKELSTSGPPHDRRFTFQVLIDEKEFGEAKGRSKTEARNAAAKLAVDILDN
Mu-PKR[b] (dsRMB2)	96-162	YIGLVNSFAQKKKLSVLIEQCEPNSELPQRFICKCKIGQTMYGTGSGVTKQEAKQLAAKEAYQKLLK
Ra-PKR[c] (dsRBM1)	10-76	VDKLNKYSQIHKVKIIYKEISVTGPPHDRRFTFQVIIEEREFPEGEGRSKQEAKNNAAKLAVEILDN
Ra-PKR[c] (dsRBM2)	96-162	YIGLVNSFAQKENLPVNFELCDPDSQLPHRFICKCKIGQTTYGTGFGANKKEAKQLAAKNAYQKLSE
Hu-TARBP[d] (dsRBM1)	11-76	ISLLQEYGTRIGKTPVYDLLKAEGQAHQPNFTFRVTV-GDTSCTGQGPSKKAAKHKAAEVALKHLKG
Vaccinia E3L	119-184	VTVINEYCQITRRDWSFRI-ESVGPSNSPTFYACVDIDGRVFDKADGKSKRDAKNNAAKLAVDKLLG
Consensus		KSLLNELAqklgiTPEYELIEEsgpAHSPEFTCEVIVGGEeFFTGSGSSKKEAKQNAAKKALEKLLK

[a]The dsRBMs of PKR from various species are compared with those in a selection of other dsRNA-binding proteins. Residues at which mutations have been shown to inhibit dsRNA binding are underlined. For more comprehensive lists of proteins containing such a motif, see references 41, 49, 55, and 58. The consensus sequence for the dsRBM motif, derived from analysis of a large number of such sequences,[58] is also shown. The most strongly conserved residues of the consensus sequence are indicated in upper case letters.

[b]Murine PKR.

[c]Rat PKR.

[d]Human Tar binding protein.

TABLE 2. CRITICAL AMINO ACID RESIDUES FOR dsRNA BINDING OR DIMERIZATION OF PKR OR BOTH

Amino acid residue number[a]	*Mutation*	*Effect on dsRNA binding*[b]	*Effect on dimerization*[b]
18-20	RQK to GAL	Complete inhibition	
19	Q to L or A	Strong inhibition	No effect
21-23	QGV to GAL	Little effect	
35, 36	PP to LL	Strong inhibition	
38-40	DRR to GAL	Little effect	
41	F to A	Complete inhibition	No effect
51-53	REF to GAL	Complete inhibition	
55	G to A	Little effect	
57	G to A	Complete inhibition	
58-60	RSK to GAL	Complete inhibition	
59	S to A	Strong inhibition	
60	K to A	Complete inhibition	No effect
61	K to Q or A	Strong inhibition	No effect
61-64	KEAK to AGAA	Complete inhibition	
62	E to A	Partial inhibition	
64	K to E or A	Complete inhibition	No effect
66-68	AAA to GAP	Complete inhibition	
68	A to D or P	Complete inhibition	Strong inhibition
69	K to A	Partial inhibition	
72	V to Y or A	No effect	No effect
73	E to A	Little effect	
75	L to A	Complete inhibition	
78-80	EKK to GAL	Partial inhibition	
108-110	IAQ to GAL	Strong inhibition	
110	Q to L or A	Partial inhibition	Partial inhibition
111-113	KKR to GAL	Strong inhibition	
131	F to A	Complete inhibition (??)[c]	Partial inhibition
134-136	KCK to GAL	Partial inhibition	
150	K to A	Complete inhibition	No effect
154	K to E	Complete inhibition	No effect
158	A to D or P	Complete inhibition	Complete inhibition
158, 159	AK to GA	Little effect	
158-160	AKL to GAL	No effect	
296	K to R	No effect	No effect

[a]Residues are numbered using the human PKR amino acid sequence.

[b]For experimental evidence of effects of point mutations on dsRNA binding, see references 39, 41, 42, 46, 47, 85, 204. For experimental evidence of effects of point mutations on PKR dimerization, see references 47, 113, 114, 223.

[c]The effects of the F131A mutation are the subject of conflicting data in the literature.[47,204]

dsRNA-binding proteins) cannot achieve such insertion. This is because the major groove of the A form of dsRNA is too narrow to allow such an interaction,[59] requiring α-helices to be inserted into the minor groove instead. It has been shown that the presence of 2′-OH groups is essential for the recognition of double-stranded nucleic acids by PKR.[60] Analysis of the binding of short dsRNA molecules to isolated segments of PKR, containing either dsRBM1 alone or both dsRBMs, has suggested that the minimal length of dsRNA that contacts each motif is about 11 base pairs.[45,60] However, optimal binding of RNA to the complete PKR molecule, necessary for activation of the kinase, requires approximately 85 bp.[61] The implications of this for the activation of PKR by dsRNA are discussed later in the review.

Organization of the protein kinase domains of PKR

Analysis of the amino acid sequences of mammalian PKRs reveals conservation of the domains common to all serine and threonine protein kinases.[35] An additional feature proposed for the kinases that use initiation factor eIF-2α as a substrate (i.e., PKR, HRI, and GCN2) was the presence of an insert between kinase domains IV and V.[35,62,63] It was originally suggested that this region is involved in substrate recognition. However, more recent studies have suggested that this insert is in fact part of an extended kinase domain, required for catalytic activity rather than substrate binding.[64] A number of reports now indicate that substrate recognition in fact involves sequences to the C-terminal side of the insert region[64,65] (T.V. Sharp and R. Jagus, personal communication).

REGULATION OF EXPRESSION OF THE *PKR* GENE

Relatively little work has been published about the regulation of expression of the *PKR* gene at the transcriptional level. The structure of the promoter[28] suggests that transcription is

likely to be controlled by a number of factors and signalling pathways in addition to the classic induction by IFNs. It has been suggested that the transcription factor IRF-1 can induce PKR,[66] possibly through activation via the ISRE element present in the 5′ flanking region of the gene. An indirect effect involving IFN production by IRF-1-overexpressing cells was ruled out in this study. Experiments involving expression of dominant negative mutants of PKR also suggest that induction of the protein kinase may be at least partly responsible for the growth inhibitory and tumor suppressor activities of IRF-1.[66] In cells that do not express IRF-1, PKR synthesis is reduced, and it has been proposed that this may contribute to the transformed phenotype in some leukemias and myelodysplasias.[67] However, IRF-1 does not appear to be essential for PKR expression, as induction by IFNs still occurs in IRF-1 knockout mice or cells derived from them.

Cells not exposed to exogenous IFNs still contain a basal level of PKR. It is not known if this reflects a constitutive rate of transcription or if low levels of inducers are essential for PKR synthesis. What is clear, however, is that synthesis can be induced 5-fold to 10-fold on treatment with IFNs.[7–9] The majority of PKR resides in the cytoplasm, and much of it is associated with the ribosomes, from which it can be removed by washing in high salt concentrations.[5,68,69] Following IFN treatment, some of the additional PKR can be found in the soluble fraction of the cytoplasm,[69] perhaps reflecting saturation of the available ribosome binding sites.[70] The protein kinase has also been detected in the nucleus, where it is localized specifically in the nucleolus.[69,71] The latter pool constitutes approximately one fifth of the total enzyme in Daudi cells before IFN treatment,[69] but the significance of nucleolar PKR remains to be established. One possibility is that it localizes to this cellular compartment because of its ability to associate with nascent ribosomes. Interestingly, in Daudi cells, the level of nucleolar PKR is not increased by IFN treatment, and this fraction of the protein kinase is relatively underphosphorylated in comparison with the cytoplasmic form. These findings suggest that nucleolar PKR may not be active *in vivo* and may play no role in the regulation of cellular function by IFNs.

The possibility that other cytokines besides IFNs may also be able to regulate PKR synthesis has not been widely investigated. Recently, evidence has been presented for the induction of PKR mRNA and protein by tumor necrosis factor-α (TNF-α) in U937 cells.[72] Another report suggests that transforming growth factor-β (TGF-β) can inhibit the expression of PKR (and other IFN-inducible genes) that occurs in association with myogenesis.[73] However, it is not known if this is mediated by a direct effect of the TGF-β signal transduction pathway on the transcription of the *PKR* gene.

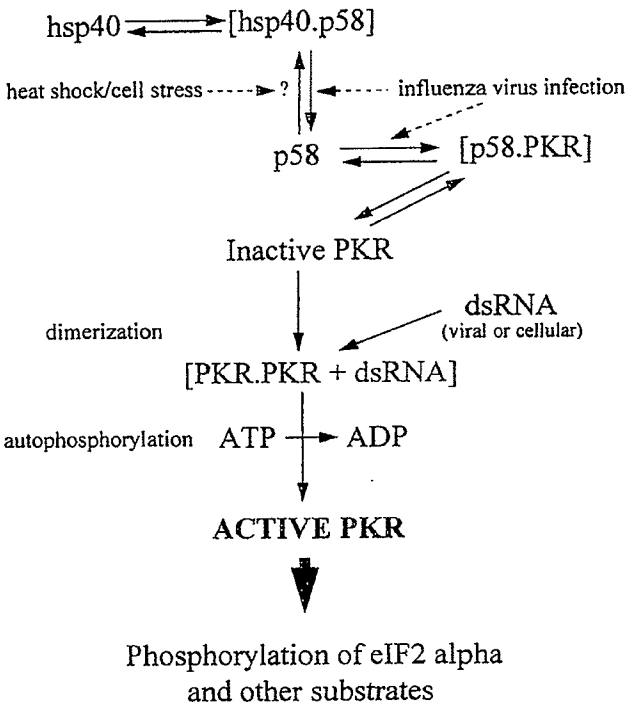

FIG. 2. Potential mechanisms of regulation of PKR in infected and uninfected cells. PKR is subject to regulation by a variety of factors in the cell. The protein kinase can be sequestered by being complexed with the inhibitory protein p58. The latter is in turn regulated by formation of a complex with an inhibitor recently identified as the molecular chaperone hsp40. Free PKR requires the binding of dsRNA to activate it in a process that involves both dimerization and autophosphorylation of the protein. Other polyanions can also activate the kinase, possibly by a different molecular mechanism. Once activated, PKR is able to phosphorylate protein synthesis initiation factor eIF-2α (and probably other substrates, such as the transcriptional regulator I-κB). This activity is independent of dsRNA. PKR may be inactivated by dephosphorylation or by reassociation with p58 or by both. Viral infection can either activate the protein kinase by providing dsRNA (although cellular forms of the latter may also be present in both infected and uninfected cells) or inhibit it by releasing free p58 (at least in the case of influenza virus infection). It is possible that the hsp40/p58 pathway is also sensitive to regulation by cellular stresses, such as those that lead to incorrect protein folding, although there is as yet little firm evidence of this. Such a mechanism (via the inhibition of p58 by hsp40) could account for the activation of PKR by stress conditions in the absence of virus infection.

MECHANISMS OF ACTIVATION OF PKR

PKR is subject to a variety of possible mechanisms by which it can be activated or inhibited (Fig. 2). It is not entirely clear how these mechanisms relate to each other or whether they represent independent pathways by which the protein kinase is controlled *in vivo*. It is possible that under normal circumstances, a substantial proportion of the PKR in the cell is maintained in an inactive state by association with one or more protein inhibitors. The best characterized of these is the protein p58, which was first identified through its involvement in the mechanism by which influenza virus prevents the activation of PKR during infection.[65,74–77] p58 may in turn be sequestered by another cellular factor, the heat shock protein hsp40.[78] PKR that is not complexed with p58 remains inactive until it associates with its activating ligand, dsRNA.

PKR that becomes bound to dsRNA acquires two potential protein kinase activities. Before it is able to act as a kinase for other substrates, the protein must undergo autophosphorylation. In fact, there is now substantial evidence that the autophosphorylation process is actually a transphosphorylation of one

PKR molecule by another. Catalytically inactive mutants of PKR can be readily phosphorylated by the wild-type enzyme *in vitro*,[79,80] but it is not certain if true intramolecular autophosphorylation can also take place. Phosphorylation occurs at multiple sites on the PKR molecule (involving at least seven different serine and threonine residues). Some of these sites have been mapped recently to two distinct regions of the protein, although other sites are also likely to exist. Autophosphorylation *in vitro* in the presence of [γ-^{32}P]ATP and dsRNA leads to labeling at positions Ser^{242}, Thr^{255}, and Thr^{258}.[81] DsRNA-dependent activation also results in phosphorylation of a cluster of sites nearer the N-terminus of PKR between the two RNA-binding domains (D.R. Taylor and M.B. Mathews, personal communication). Whereas mutation to alanine of the three amino acids in the former group of sites attenuates kinase activity, phosphorylation of the latter region seems less critical for PKR activation.[81]

The only clearly established mechanism for the activation of PKR requires the interaction of dsRNA with the enzyme. Although the exact nature of this interaction is not understood, various features of the activation process have been established. One of the major properties of PKR is that it is activated by low levels of dsRNA but inhibited by higher levels. This gives rise to a characteristic bell-shaped curve for activation of the enzyme as a function of dsRNA concentration.[2,5] Two models have been proposed to explain this phenomenon. The first suggests that activation involves two PKR molecules binding in tandem to one molecule of dsRNA and then phosphorylating each other in an intermolecular event.[82] In this model, at higher levels of dsRNA, the RNA-dependent dimerization of PKR is less likely to occur because the relative stoichiometry favors the binding of only one molecule of the kinase to one molecule of dsRNA.[82] This would impair or eliminate intermolecular phosphorylation. An alternative, earlier model for the activation of PKR suggested that there might be two distinct binding sites for dsRNA on the protein, an activating site that has a high affinity for dsRNA and an inhibitory site with low affinity for dsRNA.[83] The latter would thus only be occupied at high dsRNA concentrations. However, the presence of two functionally distinct dsRNA binding sites has largely been ruled out by more recent studies involving competitive RNA binding assays and the use of various mutant forms of PKR.[61,84]

Little information is available concerning the detailed molecular mechanism by which PKR activation takes place. It has been suggested that a conformational change in the protein occurs on the binding of dsRNA,[61,70,85] although the nature of this remains obscure. Such a change may relieve an inhibition of kinase activity imposed either by the dsRBMs themselves or by the region lying between them, thus allowing autophosphorylation to occur.[27,86] Physical studies are now being applied to test these ideas.[87] The subsequent activity of PKR as a protein kinase for other substrates is independent of dsRNA[68] and presumably is reversible only by dephosphorylation of critical sites in the PKR protein.

The nature of the dsRNAs that are responsible for PKR activation still requires considerable clarification. In virus-infected cells, viral replicative intermediates or complementary transcripts from opposite strands of viral DNA genomes may be involved,[88] although other viral RNAs can also activate the kinase. The situation is less clear in the case of uninfected cells. There is evidence that some cellular RNAs (presumably with extensive secondary structure) can activate PKR, at least *in vitro*,[40,89,90] but the exact nature of the structures required has not been established.

Double-stranded RNAs may not be the only activators of PKR in the cell. Other regulatory molecules for this protein kinase include polyanions, such as heparin and dextran sulfate.[13,43,91] The molecular mechanism by which heparin activates PKR appears to be significantly different from that of dsRNA, and the two types of activator may bind to different regions of the protein.[91] Mutant forms of the enzyme with deletions around the amino terminus, some of which are incapable of binding dsRNA, are still able to be activated by heparin[43] or other factors.[92] A number of studies have implicated one or more proteins of about 90 kDa in the regulation of PKR activation.[93–95] It is not clear if the same protein is being described in each of these reports, but if so, the data suggest that this molecule may be important in the control of the activation process. The 90-kDa protein(s) can also be phosphorylated either by PKR itself or by other kinases. One study has reported that a 97-kDa PKR-associated protein is phosphorylated on tyrosine in cells treated with IL-3 and that this correlates with the inactivation of PKR.[94]

In vivo the phosphorylation of eIF-2α is subject to regulation under a number of conditions. The ratio of phosphorylated/unphosphorylated eIF-2α increases in cells in response to a variety of stresses, such as heat shock or sodium arsenite treatment,[96,97] growth factor withdrawal,[94] or nutrient deprivation,[98,99] with a consequent inhibition of polypeptide chain initiation.[98,100] In the majority of these cases, it has not been conclusively established that PKR activation is responsible for the effects observed. However, recent evidence has shown that changes in intracellular calcium levels or distribution can regulate eIF-2α phosphorylation (reviewed in reference 101), and in this case, the data strongly support the involvement of PKR. Intracellular calcium can be released from endoplasmic reticulum stores by treating cells with calcium ionophores, such as A23187. Such treatment represents a cellular stress that results in a rapid suppression of translational initiation, and this occurs in conjunction with increased phosphorylation of eIF-2α.[102–105] Following exposure of cells to A23187, there is a twofold to threefold increase in the activity of an eIF-2α kinase, which has been shown to be PKR. Strong evidence in support of a role for PKR in the A23187-induced stress response also comes from observations that cells expressing various inhibitors of the kinase (dominant negative PKR mutants or the HIV TAR binding protein) or a nonphosphorylatable form of eIF-2α are more resistant to the effects of the calcium ionophore.[106]

It is not clear how PKR is activated during the cellular stress of calcium mobilization, but the process apparently still requires dsRNA binding. Extracts from calcium-depleted cells showed a greater degree of PKR activation when incubated in the presence of reovirus dsRNA than did extracts from control cells,[105] and *in vivo* expression of a PKR fragment containing the dsRNA-binding region was able to protect cells from activation of endogenous wild-type PKR following A23187 treatment.[106] It now appears that A23187 is just one example of a number of cellular stress-inducing agents that lead to PKR activation. A recent report indicates that physiologic insults that induce a

cytoplasmic heat shock-type (protein misfolding) response in cells, such as treatment with sodium arsenite, dithiothreitol, or *t*-butylhydroperoxide, also cause inhibition of protein synthesis through activation of PKR.[97] The mechanism of this response may involve inactivation of the PKR inhibitor, p58,[74,107] by another protein, which has recently been identified as the molecular chaperone hsp40[78] (Fig. 2). hsp40, the eukaryotic homolog of the bacterial DnaJ protein, is involved in mediating the correct folding and assembly of polypeptide chains and may thus provide a link between heat shock-type signalling mechanisms and the regulation of protein synthesis via p58 and PKR.

Alcázar et al.[108] have shown that inhibition of protein synthesis following Ca^{2+} ionophore-induced phosphorylation of the eIF-2α subunit in neuronal cells is associated with the appearance of apoptosis characteristics. This is consistent with other observations implicating catalytically active PKR in the induction of apoptosis.[109–112] Further studies are now required to investigate how eIF-2α phosphorylation or other consequences of PKR activity trigger the apoptopic mechanism following exposure of cells to physiologic stresses.

RNA-PROTEIN AND PROTEIN-PROTEIN INTERACTIONS

PKR is an enzyme that is involved in a number of regulatory interactions involving the binding of both RNA ligands and other proteins. In the case of RNA binding, this may have either a positive or a negative effect on activation of the kinase, depending on the nature of the molecule in question. In contrast, most cases so far characterized of regulatory protein-protein interactions involving PKR are associated with inhibition of kinase activity.

RNA recognition by PKR

As indicated previously, some RNA species can activate PKR, whereas others inhibit activation. Some major examples of each category are listed in Table 3. It is not yet apparent how binding occurs to bring about these opposite effects, as the two site model for stimulatory versus inhibitory RNA binding[83] can now be discounted. However, PKR exhibits differing affinities for dsRNAs of different sizes, and Green and Mathews[41] have postulated that only dsRNAs that are long enough to allow binding to both the regulatory dsRNA-binding motifs of the protein will induce a conformational change that allows activation of the enzyme. An RNA length of about 85 bp has been shown to be optimal for binding to and activation of PKR, and dsRNAs containing less than about 30 bp do not bind in a stable way to the protein.[61] This has led to the suggestion that the region of PKR between the two dsRBMs may act like a hinge. Thus, binding of dsRNA below a length of 30 bp to both motifs would add too much strain to the hinge, and interactions of such molecules can involve only one motif of PKR.

The separate affinities of the two RNA-binding motifs for various RNA ligands have been examined using purified fragments of PKR, referred to as p10 and p20, which contained one or both of the dsRBMs, respectively.[45] These studies have found that p20 is a more effective competitor for dsRNA binding than p10 in PKR activation assays, consistent with previous observations that both motifs are required for efficient binding.[41] When the length of RNA duplexes was varied, the minimal length of dsRNA required for binding to p20 was found to be as short as 11 bp, which approximates to one turn of the A-type dsRNA helix. In view of the mounting evidence that PKR dimerizes during its activation,[47,70,85,87,113,114] it seems likely that the earlier data can be reinterpreted as indicating that

TABLE 3. RNA SPECIES THAT ACTIVATE OR INHIBIT PKR

RNA	*Description*	*Function of RNA*	*References*
Activators			
Reovirus s1 mRNA	Nt 416-572 or 577-1462 of the mRNA are sufficient	mRNA for σ1 protein	115, 116, 197
HIV-1 TAR RNA[a]	82 nt sequence present in 5′ regions of HIV-1 mRNAs	Interacts with HIV-1 Tat	132, 133, 195
HTLV-1 Rex response element	Target sequence for HTLV-1 Rex protein	Regulation of splicing	196
Human α-tropomyosin mRNA	3′ untranslated region (515 nt starting at termination codon)	mRNA for α-tropomyosin	89
Hepatitis delta agent[b]	Nucleotides 482-963 are sufficient	Genomic sequence	117, 118
EBV W repeat	198 nt repeat present in the 5′ regions of several EBV mRNAs	Attenuation of translation?	119
Potato spindle tuber viroid RNA	Plant viroid with pathogenic effects	Activation of plant PKR during viroid infection?	198
Adenovirus VA_I	Small viral RNA inhibitors produced at high levels	Maintenance of translation	123
EBV EBER-1	Abundant small viral RNA present in EBV-infected cells	Assists transformation(?)	121
Hepatitis delta agent[b]	Nucleotides 482-963 are sufficient	Genomic sequence	117, 118

[a]The ability of TAR RNA to function as a PKR activator, as well as an inhibitor at higher concentrations, is the subject of conflicting reports in the literature.[130,132,133,195]

[b]This RNA functions as a PKR activator in assays of protein kinase activity but is an apparent inhibitor of PKR in protein synthesis assays.[117]

30 bp are required to accommodate a PKR dimer, whereas 11 bp can only accommodate one dsRBM on one PKR molecule.

Because the precise nature of the RNA secondary structure required for recognition by PKR has yet to be elucidated, no universal model can be described that predicts which RNAs will be good ligands and which will activate or inhibit the kinase. Some viral RNAs with extensive secondary structure, such as reovirus s1 mRNA,[115,116] hepatitis delta RNA,[117,118] and transcripts from the Bam HI W repeat region of the Epstein-Barr virus genome,[119] are potent activators. However, these molecules are unlikely to contain 30 or more uninterrupted base pairs, and it seems probable that elements of tertiary structure (e.g., pseudoknots) are also important for PKR recognition and activation.[118]

Much work has been done on other virally encoded RNAs that are known to inhibit the kinase (reviewed in references 120 and 121). A molecule that has been a major focus of such research is the adenovirus small RNA VA_I[122] (reviewed in reference 123). Various regions of the VA_I RNA structure have been implicated as having a role in binding to and inhibiting PKR.[44,124–128] Studies have suggested that the dsRBM1 of PKR can interact both with the minor groove of the apical stem of VA_I and with the central domain of the RNA.[44,129] Other small viral RNAs with an ability to inhibit the activation of PKR by dsRNA include EBER-1 of Epstein-Barr virus[84,121] and the TAR RNA of HIV-1.[130] There is little doubt that at high concentrations these RNAs, like VA_I, are PKR inhibitors, although relatively few structural investigations of their modes of interaction with the kinase have been carried out to date. What is controversial, however, is the possibility that such small RNAs, at lower concentrations, may also be PKR activators. The argument centers around the technical problem that RNA molecules synthesized by *in vitro* transcription, unless rigorously purified, are commonly contaminated with low levels of nonspecific dsRNA transcripts synthesized by the bacteriophage RNA polymerases employed.[131] Mathews' laboratory has argued that the apparent ability of TAR to activate PKR is an artifact caused by this problem.[130] Other groups, however, have shown that a specific structural feature, that is, the integrity of the base-paired stem of TAR, is essential for stimulation of PKR activity[132] and that RNA synthesized chemically rather than enzymatically retains the ability to activate the kinase.[133] Neither of these observations can be explained by the dsRNA contamination hypothesis.

The affinities of several of these small viral RNAs for PKR have been measured by a variety of methods.[45,84,134] The results vary by several orders of magnitude (Table 4), perhaps reflecting not only differences between ligands but also differences in the methods of measurement. However most measurements give relatively low K_d values (in the nM range), indicating tight binding of these RNA regulators to the protein.

Homodimerization and heterodimerization

There is now a substantial body of evidence indicating that PKR can undergo protein-protein dimerization, both with itself and with other proteins (see reference 135 for a review of the earlier literature on this subject). Dimerization of PKR is likely to be required for activation,[70,85,87,113] although there is controversy over whether this is mediated by dsRNA acting as a bridge between two PKR molecules[86] or if direct protein-protein interactions are necessary or sufficient.[87] Interpretation of experiments using mutants of PKR has been made difficult by the fact that the same regions of the protein apparently are required for both kinds of interaction.[47,85,114] Furthermore, the proteins with which PKR can form heterodimers may themselves be RNA-binding proteins. An example is the cellular TAR-RNA binding protein.[114,136] It would seem an unlikely coincidence that such protein partners, which can themselves individually interact with structured RNA ligands, form complexes that do not obligatorily include such RNA molecules. Nevertheless, there are reports that some mutant forms of PKR that lack the ability to bind dsRNA not only are still able to dimerize *in vivo* and *in vitro*[47] but also can be activated *in vivo*.[86,92] The most likely explanation for the latter finding is that these molecules are phosphorylated by endogenous wild-type PKR via direct protein-protein interactions. Thus, although the same region of the protein contributes to both dsRNA binding and homodimerization, different amino acid residues within this domain may be more important for one or other of these properties (Table 2).

The fact that one PKR molecule can phosphorylate another would explain why heterologous partners in a dimer, such as the TAR-RNA binding protein, act as inhibitors of the kinase.[137] An alternative explanation of the latter phenomenon, however, is that such other RNA-binding proteins compete with PKR for limiting amounts of dsRNA. This explanation was fa-

TABLE 4. AFFINITIES OF RNA LIGANDS FOR PKR *IN VITRO*

RNA species	*Estimated K_d*	*Method of determination*	*Reference*
poly(I).poly(C)	0.35 and 1 nM	Filter binding assay	83
poly(I).poly(C)	2 nM	Gel shift analysis	134
85 bp dsRNA	4 nM	Gel shift analysis[a]	45
Adenovirus VA_I	0.3 nM	Filter binding assay	84
Adenovirus VA_I	1–4 nM	Gel shift and competition analysis	134
Adenovirus VA_I	350 nM	Gel shift analysis[a]	45
EBV EBER-1	0.3 nM	Filter binding assay	84
EBV EBER-2	0.3 nM	Filter binding assay	84
HIV-1 TAR	200 nM	Gel shift and competition analysis	134

[a]Data relate to assays conducted with an N-terminal fragment of PKR (amino acids 1–184) containing both dsRBMs.

vored by Wu and Kaufman in a study of PKR activation using transfected cells that expressed various PKR inhibitors.[138]

The dominant negative mutant controversy

In view of the alternative interpretations of the data about the regulation of PKR by other dimerization partners, it is perhaps not surprising that considerable controversy has raged over the mechanism of action of mutant forms of PKR as inhibitors of the wild-type enzyme. Understanding this mechanism is particularly important because PKR has been implicated as a tumor suppressor protein[139,140] (see later in this review), and it has been proposed that certain mutant forms of the kinase are tumorigenic because they exert a dominant negative effect on the wild-type enzyme.

The mechanism by which PKR mediates a tumor-suppressing phenotype is not yet clear, but several possibilities can be proposed to explain the reversal of the effect by PKR mutants (Fig. 3). First, the presence of wild-type and mutant forms of the protein in a cell could give rise to a situation where both species compete for binding of limited amounts of dsRNA activators [pathway (a) in Fig. 3]. This explanation is consistent with the fact that whereas expression of wild-type PKR is growth inhibitory for the yeast *Saccharomyces cerevisiae*, mutations in the dsRNA-binding domains abrogate this effect.[141] Furthermore, coexpression of only the dsRNA-binding region of PKR was able to relieve the inhibitory effect of the wild-type enzyme in yeast.[63] Competition for dsRNA between wild-type and mutant PKR has been clearly demonstrated *in vitro*, where the inactive point mutant K296R was able to inhibit both autophosphorylation and phosphorylation of eIF-2α mediated by wild-type PKR and could rescue protein synthesis from inhibition by dsRNA.[142–144] Moreover, these effects could be reversed simply by adding more dsRNA to the assays. This indicates that the dominant negative effect exerted by this particular mutant PKR on the wild-type kinase is due only to competition for dsRNA binding, at least *in vitro*. The K296R mutant can also inhibit the activation of another dsRNA-dependent enzyme, 2′5′-oligoadenylate synthetase, and again this effect is reversed at higher concentrations of dsRNA in a cell-free system.[143] In view of these data, it is possible that the tumorigenic effect in 3T3 cells of overexpression of K296R[140] is a result of inhibition of other pathways requiring dsRNA *in vivo*. However, such a hypothesis is difficult to test, as attempts to reverse the effect of K296R would require long-term elevation of dsRNA levels in the cell.

Another possible mechanism for a dominant negative effect is that inactive mutant PKR molecules bind unproductively to the enzyme's normal substrates and protect them from phosphorylation by the wild-type kinase [pathway (c) in Fig. 3]. This model is less favored because, at least in the case of the K296R mutant, it would not explain the reversal of the inhibition by higher concentrations of dsRNA. In addition, the activity of another protein kinase, the hemin-regulated enzyme HRI, which phosphorylates the same site on eIF-2α, was not inhibited by PKR K296R.[142]

In view of the mounting evidence that dimerization of wild-type and mutant species of PKR can occur *in vitro* and *in vivo*, the currently most favored explanation for dominant negative

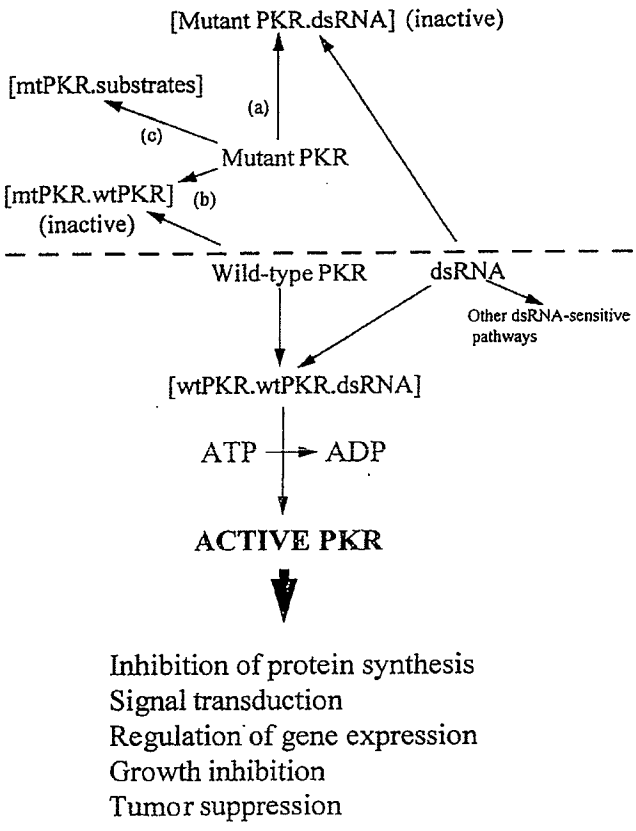

FIG. 3. Summary of possible mechanisms of action of dominant negative mutants of PKR. The lower part of the figure (below the dashed line) shows the normal mechanism of activation of PKR by dsRNA and the biologic consequences of such activation for protein synthesis, signal transduction, transcriptional control, cell growth, and tumor suppression. The upper part illustrates three possible pathways by which mutant forms of PKR may interfere with the functions of the wild-type enzyme. These pathways are not necessarily mutually exclusive. In pathway (a), the mutant acts by competing with wild-type PKR for limiting amounts of dsRNA activators. Note that this pathway might also lead to inhibition of other dsRNA-requiring enzymes, such as the 2′5′-oligoadenylate synthetases. In pathway (b), inactive heterodimers are formed between mutant and wild-type proteins (these complexes may also contain dsRNA; not shown). Pathway (c) suggests binding of mutant PKR to substrates of the kinase, thus protecting these proteins from phosphorylation by the wild-type enzyme. The current evidence most favors pathway (b), although there is also some *in vitro* evidence for pathway (a). See the text for further details.

effects is one invoking heterodimerization between the two molecular forms by direct protein-protein interactions[85] [pathway (b) in Fig. 3]. This would account for the tumorigenic effects of some PKR mutants that cannot bind dsRNA.[144,145] Moreover, in yeast expressing wild-type PKR, some catalytically inactive PKR mutants were dominant negative, but others (including K296R) were not, although all could bind dsRNA equally well.[85] It was proposed that in yeast, dsRNA is not limiting for the activation of PKR. Note, however, that not all forms of PKR that retain the ability to dimerize have a domi-

nant negative effect and that retention of dsRNA-binding ability may be a more important determinant of this property.[47] It is possible that these discrepancies in the literature arise from the use of different assays for the inhibition of wild-type PKR by inactive mutant forms of the kinase.

Further support for the heterodimerization model comes from experiments where PKR mutants lacking functional dsRNA binding domains were expressed in a transfection-infection system using vaccinia virus.[92] Constructs with deletions of amino acids 233-271 showed no translational inhibitory effect following infection, whereas those with mutations in the dsRNA binding domain remained active. The suggested explanation is that the latter proteins become activated as a result of phosphorylation by endogenous wild-type PKR. Mutants that have lost the ability to bind dsRNA have been reported to be partially transdominant over wild-type PKR in a reporter gene assay in mammalian cells,[46] indicating that they act by a mechanism other than competition for dsRNA.

SUBSTRATES PHOSPHORYLATED BY PKR

eIF-2α

Classically, the smallest (α) subunit of protein synthesis initiation factor eIF-2 has been considered to be the principal substrate of PKR *in vivo*. In this respect, PKR is a member of a small family of eIF-2α kinases that play important roles in the translational regulation of gene expression.[15–17,20] Initiation factor eIF-2 is responsible for binding the initiating methionyl-tRNA (Met-tRNA$_f$), together with a molecule of GTP, and placing the Met-tRNA$_f$ on native 40S ribosomal subunits (reviewed in reference 146). During the course of this process, the GTP is hydrolyzed to GDP and inorganic phosphate, and when the eIF-2 leaves the ribosome later in initiation (at the 60S subunit joining stage), it does so as an inactive [eIF-2.GDP] complex. Regeneration of active eIF-2 requires the exchange of the GDP for a new molecule of GTP, catalyzed by the guanine nucleotide exchange factor eIF-2B.[146] When eIF-2 becomes phosphorylated by PKR (at the specific residue Ser^{51} on its α subunit), the initiation factor acquires an increased affinity for eIF-2B, thus sequestering the latter in an inactive complex. As a result, the rate of guanine nucleotide exchange on both phosphorylated and unphosphorylated eIF-2 falls, as eIF-2B is present in cells at less than stoichiometric levels with respect to eIF-2, and polypeptide chain initiation is slowed. This has effects on the global rate of protein synthesis, but it may also selectively inhibit the translation of specific mRNAs that, for various reasons, have a greater than average requirement for active eIF-2 in the cell. Some of these mRNAs may have particularly important roles in the control of cellular growth or differentiation.

The importance of controlling protein synthesis at this level of polypeptide chain initiation is evident from the large number of physiologic situations in which eIF-2α is phosphorylated[16] and also from the widespread existence of PKR homologs (and other protein kinases with the same specificity). A plant PKR has been described that phosphorylates plant eIF-2α,[18,19] and extensive biochemical and genetic studies have characterized the yeast enzyme GCN2 that has the same substrate specificity as PKR, although it is differently regulated.[62] One recent development is the realization that PKR may be a critical molecule for the downregulation of translation associated with the cellular response to stress (see earlier in this review). For example, two groups of agents that induce either endoplasmic reticular (glucose-regulated) or cytosolic (heat shock-type) stresses both activate pathways that ultimately converge on PKR to inhibit protein synthesis.[97,102,103]

Consistent with its major role in the regulation of polypeptide chain initiation, PKR is largely a ribosome-associated protein *in vivo*.[9,69] This may allow it to come into close proximity with eIF-2, as well as potential dsRNA activators (e.g., mRNA species with extensive secondary structure). There is evidence that the dsRBM sequences of PKR are required for the ability of the protein to bind to 40S ribosomal subunits, and it is possible that the ribosome itself plays a role in the regulation of PKR activation or activity.[147]

Other substrates

In spite of the fact that eIF-2α is undoubtedly an important substrate, it is unlikely to be the only target for PKR. The multiple biologic effects of the kinase, described in detail later in this review, suggest that other substrates also exist that could be equally important. Indeed, there is a growing amount of experimental evidence for such alternative substrates.

PKR has been shown to be an important regulator of the NF-κB family of transcription factors, and it has been suggested that control is mediated by direct phosphorylation of the NF-κB inhibitor, I-κB. *In vitro*, the latter can serve as a substrate for PKR,[148] with phosphorylation occurring in the C-terminal part of the protein (B.R.G. Williams, personal communication). However, the stoichiometry of phosphorylation may be low, and it is not yet clear whether I-κB is an efficient PKR substrate in the cell. Rather, the requirement for PKR for the activation of NF-κB by dsRNA (and some other regulators) *in vivo*, as demonstrated using selective ablation of the mRNA for PKR[149] or by the use of cells from PKR knockout mice,[150,151] may reflect the existence of a kinase cascade in which PKR is an intermediate and is not proof of a direct effect of the enzyme on I-κB.

There is some evidence that PKR can regulate the activity of other transcription factors, although again in some cases the effects may be indirect. Koromilas et al.[152] have shown that the protein kinase is able to modulate transcription of the immunoglobulin κ gene in a manner that is apparently independent of NF-κB regulation. Recent data point to the association of PKR with the Stat1 component of the IFN-activated transcription factor complex ISGF-3 in a manner that correlates inversely with the DNA binding activity of Stat1.[153] Another IFN-sensitive factor, IRF-1, is also subject to control by PKR, as its function is impaired in cells lacking PKR or expressing dominant negative mutants of the kinase,[66,111] but direct phosphorylation of IRF-1 by PKR has not yet been reported. Interestingly, IRF-1 may itself induce PKR expression, implying an important positive feedback loop acting at the level of gene transcription.

The HIV-1-encoded Tat protein interacts with PKR *in vivo* and *in vitro*, and both the one and two exon forms of Tat can be a substrate for the dsRNA-regulated protein kinase *in vitro*.[154,155] It may be significant for the strategies used by

HIV-1 to regulate PKR during infection that Tat can block both the activation of the kinase and the ability of the latter to phosphorylate eIF-2α in cell-free systems. This inhibition probably involves direct Tat-PKR association,[155] but it may also result from the ability of the viral protein to bind TAR and perhaps other RNA activators of the kinase.[156]

BIOLOGIC EFFECTS OF PKR

In recent years it has become increasingly evident that PKR is involved in a wide range of biologic effects, both before and after exposure of cells to IFNs. It is an important mediator of many of the antiviral effects ofthe IFNs and, together with the IFN-inducible 2′5′-oligoadenylate synthetases,[157] contributes to the shutoff of protein synthesis following virus infections.[158,159] In many cases, viral dsRNAs are probably the activators of this process. However, it is clear that PKR can exert effects in uninfected cells also and that it can be a potent growth inhibitory protein when activated. It is not known which function may have arisen first during the course of evolution; that is, whether the antiviral activity of PKR represents a specialized aspect of a more general effect on gene expression or the protein kinase initially evolved as a cellular defense mechanism, which then acquired other functions operating in the absence of virus infection.

Antiviral actions

The creation of cell lines that constitutively express PKR[160] or, conversely, that are defective in PKR activity or contain an unphosphorylatable form of eIF-2α[161,162] has established the importance of the protein kinase for mediating some of the antiviral effects of IFNs. Furthermore, the data largely support a role for PKR-mediated phosphorylation of eIF-2α in this antiviral activity. However, different viruses vary in their susceptibility to PKR. For example, replication of encephalomyocarditis (EMC)[160] and vaccinia virus[163] is sensitive to the level of activity of the kinase, whereas vesicular stomatitis virus replicates normally in the presence of PKR. Such variations may reflect differences either in the ability of viruses to activate the protein kinase during the course of their replication or in the ability of viruses to block the actions of PKR in infected cells. A well-studied example of a mechanism resulting in inhibition of PKR function is the synthesis of the small VA_I RNA by adenoviruses.[123] Other viruses employ a variety of different strategies to achieve the same end.[120] One report also suggests that the large T antigen of SV40 virus somehow overcomes the translational block normally imposed by the phosphorylation of eIF-2α,[164] although how this is achieved remains unclear.

Control of cell proliferation and differentiation

Although many earlier studies of PKR focused on the role that the kinase plays in virally infected cells, we now know that there is a role for PKR in uninfected systems also (reviewed in references 23, 165, 166). This function centers around the ability of the enzyme to control cell proliferation.[22] Initial observations[167,168] indicated that PKR activity can vary according to the state of growth of mammalian cells in culture, and recently this has been correlated with changes in eIF-2 and eIF-2B function.[169] Expression of wild-type mammalian PKR in yeast inhibits cell proliferation.[63] The growth-inhibitory effects of the transcription factor IRF-1 in NIH 3T3 cells also require the presence of active PKR.[66] However, it is still not clear to what extent the well-established ability of changes in serum or nutrient availability to regulate protein synthesis and cell growth[170] can be attributed to phosphorylation of eIF-2α by PKR.

Some of the effects of cell growth factors, such as PDGF[171] and IL-3,[94] may be mediated by changes in PKR activity. For example, deprivation of a murine IL-3-dependent cell line of this cytokine stimulated autophosphorylation of PKR and the phosphorylation of eIF-2α and led to inhibition of protein synthesis. Following IL-3 treatment, which reversed these changes, the PKR became associated with a 97-kDa tyrosine phosphorylated protein that could be coimmunoprecipitated by anti-PKR antibodies.[94] In some situations, the changes in PKR associated with cytokine-mediated cell growth regulation may also have effects on the state of differentiation of cells. For example, the induction of myoblast differentiation and the inhibition of this process by TGF-β in a myogenic cell line were associated with increases and decreases, respectively, of PKR activity.[73] There is insufficient information to say whether PKR is a widespread mediator of cell differentiation in other systems.

Signal transduction and transcriptional regulation

As indicated, PKR has been implicated in the mechanisms by which certain growth factors and cytokines mediate their effects on target cells (reviewed in reference 24). In the case of PDGF, this requirement is somewhat paradoxical, as this growth factor is mitogenic but PKR itself is associated with inhibition of cell proliferation. The resolution of this paradox may lie in the nature of the genes that are induced by PDGF using a PKR-dependent pathway. Several growth-related genes (such as *c-fos, c-myc,* and JE) are inducible at the transcriptional level by both IFN and PDGF treatment (as well as by dsRNA itself). Induction of *c-fos, c-myc,* and JE by PDGF is blocked by 2-aminopurine (2-AP), a PKR inhibitor, suggesting a role for PKR (or at least a related 2-AP-sensitive kinase) in this transcriptional response.[171] This aspect of IFN-induced or PDGF-induced signalling can be blocked by expression of an oncogenic form of the *ras* gene, which induces a cytoplasmic inhibitor of PKR.[172] However, to date there is little information about the molecular basis for the PKR requirement for growth factor signalling.

The use of cells expressing dominant negative forms of PKR or derived from PKR knockout mice has demonstrated a requirement for PKR in the signalling pathways by which IFN-γ exerts its effects on expression of genes, such as those encoding IRF-1, the guanylate-binding protein, or immunoglobulin κ light chain.[151,152] These genes are also unresponsive to dsRNA induction in knockout cells. Moreover, establishment of the antiviral effect of IFN-γ (but not that of IFN-α) against EMC virus requires an intact PKR pathway.[150,161]

Considerable evidence now exists for transcriptional upregulation of specific genes by mechanisms requiring PKR activ-

ity. Examples include the IFN-α and IFN-β genes themselves,[161] as well as genes encoding the γ subunit of ISGF-3,[173] the vascular endothelial cell adhesion molecule VCAM-1,[174] class I MHC,[150] the guanylate-binding protein,[175] inducible nitric oxide synthase, and the FAS antigen[176] (B.R.G. Williams, personal communication). Many of these genes are inducible by dsRNA treatment of cells without a need for the production of IFN. A recent report[177] suggests that PKR may have a negative effect on the expression of the CD4 gene, at the transcriptional level, in T lymphocytes.

The involvement of PKR in induction of type I IFN gene expression at the transcriptional level is of particular interest because it potentially constitutes the basis of a positive feedback loop in the activation of the IFN system. The molecular basis for the effects of dsRNA on IFN induction has been widely studied, and regions in the promoter that are responsive to NF-κB,[178–180] IRF-1,[181] and other factors[182] have been identified. As indicated earlier, both NF-κB and IRF-1 are targets for regulation by PKR. Nevertheless, neither NF-κB expression[183] nor the presence of PKR itself[150] is essential for type I IFN induction. Interestingly, in cells from knockout mice, the PKR requirement for this process can be circumvented by prior treatment of the cells with IFN (priming), suggesting that an alternative pathway that is sensitive to dsRNA can be induced by IFN. Whether this reflects the existence of a second PKR-like gene or an entirely different mechanism remains to be established.

Participation of PKR in the transcriptional control of many genes can be explained in part by the ability of the kinase to activate members of the NF-κB family. Thus, expression of genes containing NF-κB response elements is enhanced when such genes are cotransfected into cells with a wild-type PKR expression vector.[184] In PKR-deficient cells,[149,150] in which genes that are normally induced by dsRNA or IFNs are unresponsive to these agents, inducibility can be restored if PKR is cotransfected into the cells. Although the lack of responsiveness has been attributed to deficiencies in the activation of both NF-κB and IRF-1 in these knockouts,[111,151] other transcription factors may also turn out to be regulated directly or indirectly by PKR. For example, interactions between the B lymphocyte and macrophage-specific factors PU.I and NF-EM5, or the DNA-binding ability of these factors, may be regulated by the phosphorylation of PU.I, and PKR has been proposed as a possible candidate kinase for the latter process.[152] This may account for the reported PKR requirement for activation of macrophages by lipopolysaccharide.[185] PKR-dependent induction of Fas antigen gene expression following virus infection or dsRNA treatment may involve phosphorylation of the transcription factor NF-IL-6.[186] The involvement of distinct dsRNA-activated transcription factors[187] would explain the ability of dsRNA to induce certain genes that also happen to be responsive to IFNs without the need for IFN production or the activation of the IFN-regulated ISGF-3 factor complex.[188,189]

Tumor suppression and induction of apoptosis

As might be predicted for a protein that inhibits cell proliferation, several studies have now implicated PKR as a tumor suppressor gene product (reviewed in references 165 and 166). Nude mice injected with NIH 3T3 cells expressing various inactive mutant forms of PKR develop tumors within a relatively short time. The mutant forms of PKR that have been shown to be tumorigenic are those with a deletion of amino acids 361-366 in kinase subdomain V (PKRΔ6),[139] a single amino acid substitution of arginine for lysine at the invariant position 296 (PKR K296R),[140] or a large deletion of the first 97 amino acids of the protein (including dsRBM1).[145] Cells expressing wild-type PKR do not generally induce tumor growth in mice. In one instance, tumor growth was observed in two mice after a period of 50 days,[140] but subsequent analysis of cells recovered from the tumors revealed that the PKR they expressed had no kinase activity, suggesting that a naturally occurring mutation in the protein may have been responsible for the tumorigenesis. The loss of tumorigenicity of human glioblastoma cells transfected with an IFN-α gene was associated with constitutively upregulated expression of PKR.[190] These data collectively suggest that PKR suppresses cell growth and the tumorigenic phenotype and that mutant forms of PKR have a dominant negative effect on the activity of the wild-type protein, thus abrogating its growth inhibitory and tumor-suppressing functions.

In contrast to these findings, studies with PKR knockout mice to date have shown no evidence of increased spontaneous tumorigenesis in the absence of the kinase.[150] This raises doubts concerning the explanation for the previous observations and suggests that the mere absence of PKR activity may not be sufficient for cell transformation. It is possible that the dominant negative effects of PKR mutants are exerted through one or more pathways that are not affected by loss of expression of the wild-type PKR gene or that a similar tumor suppressor function is maintained by an alternative cellular component. In addition to these considerations, loss of PKR function may constitute only one step in a multistep pathway to tumorigenesis.

The molecular basis for the dominant negative and tumorigenic effects of PKR mutants remains controversial. It seems likely that direct formation of heterodimers between active and inactive enzyme molecules may play a role, as one form of PKR that is tumorigenic has no dsRNA binding activity *in vitro*.[145] However, this mutant does not in fact exert a dominant negative effect on endogenous PKR activity in the reticulocyte lysate system,[144] raising the possibility that it functions as an oncogenic protein by virtue of an entirely different mechanism. In the case of PKR mutants that retain dsRNA binding activity, the suggestion has been made that these proteins may inhibit the activity of other dsRNA-requiring pathways with tumor-suppressing effects.[143] Set against these arguments are the observations that the (presumably) specific protein inhibitors of PKR, p58 and the TAR RNA-binding protein, as well as a non-phosphorylatable form of the PKR substrate eIF-2α, are also tumorigenic when overexpressed in NIH 3T3 cells.[136,191,192] Furthermore, the cell growth inhibitory effect of the tumor suppressor protein IRF-1 is impaired in PKR-deficient cells[66] (B.R.G. Williams, personal communication).

Whatever the mechanism(s) by which the mutants act, it should be emphasized that no direct evidence exists to support the notion that PKR functions as an antioncogene product under natural conditions. To establish a definite role for PKR in tumorigenesis, it will be necessary to show a direct link between alterations in the structure or function of the PKR gene (or that of its cellular regulators) and the pathogenesis of naturally occurring cancers.

There are reports that oncogenic proteins can interact with the pathways by which PKR acts to control cell growth. Cells transformed by an oncogenic form of the *ras* gene contain one or more endogenous inhibitors of PKR.[172,193] A recent study[194] has linked the level of PKR activity with the ability of cells to express the c-myc protein. The well-established ability of IFN to downregulate c-myc expression in myeloid leukemic cells is abolished by either of the tumorigenic PKR mutants K296R or PKRΔ6, and, conversely, ectopic expression of a deregulated form of c-myc rescues cells from the growth inhibitory effects of wild-type PKR. These data suggest that PKR may be an upstream regulator of c-myc expression (probably acting at the translational level). However, the effects of IFN on other cell growth regulatory pathways, for example, the dephosphorylation of the pRB tumor suppressor protein and downregulation of cyclins D and A, were not altered by mutant PKR expression,[194] indicating that the protein kinase only regulates a subset of the signal transduction mechanisms by which the IFNs inhibit cell proliferation.

A further twist to the story concerning growth inhibition by wild-type PKR and cell transformation by inactive forms of this kinase arises from evidence that PKR can induce apoptosis in certain cell types. Infection of HeLa cells with a recombinant vaccinia virus expressing wild-type PKR under an inducible promoter resulted in apoptosis following induction. In contrast, the K296R mutant did not cause apoptosis when similarly expressed.[109] Indeed, the latter can protect cells from apoptosis following influenza virus infection, perhaps by blocking the induction of the FAS antigen.[110,176] Recent data[72,111,112] suggest that PKR may be involved in the induction of apoptosis by dsRNA as well as by TNF-α and other agents. Thus, the ability to activate cell death under appropriate conditions and the corresponding loss of this potential in the presence of dominant negative mutants or other PKR inhibitors may be important facets of the tumor-suppressor activity of this protein kinase.

PROTEIN AND RNA REGULATORS OF PKR

In view of the wide range of biologic effects of PKR on both viral and cellular functions, it is perhaps not surprising that a variety of molecules have been identified that can regulate the activity of this protein kinase, in uninfected as well as infected cells (reviewed in reference 25). What is perhaps more unexpected is that we know far more about the nature of the inhibitors of PKR than about the activators. In view of the dsRNA dependence of PKR activation, it has widely been assumed that virally encoded dsRNA molecules are necessary cofactors for kinase function in infected cells. Although this is likely to be correct in some cases, it begs two important questions: what are the activating molecules for viruses that do not produce dsRNA-containing replicative intermediates and how may PKR be activated in the absence of virus infection? There also remain the important issues of what specific RNA structures are necessary (or sufficient) for PKR activation and what distinguishes activatory from inhibitory molecules.

Some progress has been made recently in identifying individual viral transcripts that have the potential to activate PKR (at least *in vitro* and in some cases *in vivo* also). Well-characterized examples of small RNAs that can stimulate PKR activity include the TAR sequence of HIV-1,[132,133,195] the Rex response element of HTLV-1,[196] the collapsed circular single-stranded RNA of hepatitis delta virus,[117] the s1 mRNA of reovirus (nucleotides 416-576),[116,197] the 198 nt W1W2 exon pair transcribed from a repeated region of the Epstein-Barr virus genome,[119] and potato spindle tuber viroid RNA[198] (Table 3). Care has to be taken in interpreting data on PKR activation by these RNAs *in vitro* if bacteriophage RNA polymerases have been used to generate the transcripts, as there is a potential for artifactual production of dsRNA by such enzymes.[130,131] Nevertheless, when rigorous precautions are taken to remove such contaminants[117,119] or when the RNA is synthesized by chemical means,[133] activation of PKR can still be observed in several of the cases, suggesting that these molecules are true activators of the kinase. Another viral activator that is still in the process of characterization comprises sequence(s) spanning the Us11 and Us12 genes of herpes simplex virus-1.[95,199] What is striking about some of these RNA species is that they do not need to contain extensive stretches of uninterrupted double-stranded regions to function effectively as PKR activators (see, for example, reference 117). Determination of the actual RNA structural requirements, in terms of secondary and tertiary interactions,[118] both for high-affinity binding to PKR and for the ability subsequently to activate the enzyme, will be an important challenge in this field.

By comparison with the viral activators, far less is known about cellular RNAs that can bind to and stimulate PKR. Early observations suggested that poly(A)-containing RNA preparations from a range of cell types contain molecules that can activate the kinase *in vitro*,[90] but characterizing individual species with this ability has proved very difficult. Candidates for cellular RNAs with PKR activation ability include part of the 3′ untranslated region (UTR) of α-tropomyosin mRNA,[89] the 5′-end of IFN-γ mRNA (R. Kaempfer, personal communication), the mRNA for the growth-regulated protein p23 (U-A. Bommer, A. Elia, M. James, and M.J. Clemens, unpublished observations), the small cytoplasmic Y RNAs of unknown function (M. James, G. Pruijn, and M.J. Clemens, unpublished observations), and possibly the mRNA for PKR itself.[200] One caveat with respect to such studies is that *in vivo* all of these RNAs will undoubtedly be complexed with proteins and may not assume the same conformations as when deproteinized *in vitro*. Nevertheless, the phenotypic consequences of expressing the 3′ UTR of α-tropomyosin mRNA in rhabdomyosarcoma cells are consistent with possible activation of PKR *in vivo*.[201] Again, this RNA is largely single-stranded.[89]

There are a small number of extensively studied short viral RNAs that are PKR inhibitors (reviewed in reference 121), of which the most thoroughly characterized is the VA_I RNA of adenovirus.[93,122–124,126,128] Our laboratory has shown that the EBER-1 (and perhaps EBER-2) RNAs of Epstein-Barr virus are also inhibitors of the kinase.[84,121,202] These molecules are only effective *in vitro* at relatively high concentrations, but this is compatible with a physiologic function for them in the cell, as they are abundantly expressed late in infection (or, in the case of the EBERs, constitutively during latent infection of the host cells). Whether VA_I and the EBERs can also function as PKR activators at lower concentrations (in accord with the bell-shaped curve for activation of PKR by dsRNAs) is still con-

troversial. Gel-purified VA_I only functions as an inhibitor of PKR,[131] but it is possible that gel purification under denaturing conditions may alter the conformation of the RNA to preclude its ability to activate the kinase.[203] The concept of alternative conformations of a given RNA molecule that cause activation and inhibition, respectively, has been proposed in the case of hepatitis delta RNA.[117] If correct, this would imply that PKR requires different structural features in an RNA ligand for activation or inhibition, even though the same binding site on the protein appears to be involved in both types of interaction.[40,45,84,204]

With one exception (the capsid protein of Semliki Forest virus),[205] there are no published examples of proteins that can activate PKR. In contrast, a variety of protein inhibitors of the kinase, of both viral and cellular origin, are known (reviewed in references 135 and 206) (Table 5). In the case of the viral inhibitors, these are important for blocking the activity of PKR in infected cells (see references 120 and 158 for discussions of the strategies used by viruses to inhibit PKR). The endogenous cellular inhibitors are likely to be involved in preventing the inappropriate activation of PKR by RNA activators constitutively present in cells, and they may be particularly important in controlling PKR activity during the regulation of cell growth or apoptosis.[207] In at least one case, a cellular inhibitor, the protein p58, is also recruited by a virus (influenza) to block activation of PKR during infection,[75,76,208] although it is not yet clear exactly how this is achieved.

Aside from the possibility that dephosphorylation of PKR by one or more protein phosphatases can inactivate it,[209,210] a phenomenon that has been studied remarkably little in recent years, the mechanisms of action of the protein inhibitors of PKR fall broadly into two categories. These are the sequestration of PKR activators as a result of the ability of the inhibitor protein to bind dsRNA[50,52,211] and direct binding of the inhibitor to the kinase via protein-protein interactions. Examples of both classes are listed in Table 5. Of particular interest is a gene product of vaccinia virus, K3L, which is believed to inhibit PKR by acting as a pseudosubstrate.[212–214] The region of the kinase necessary for interaction with K3L (and by extrapolation with eIF-2α) has been identified recently as amino acids 366-415.[64] In contrast, the region of PKR that interacts with the cellular protein inhibitor p58[74,76,77,191,208] involves amino acids 244-296.[65]

Proteins that may act against PKR by virtue of their RNA-binding properties (including possible competition for dsRNA binding) are of interest for a number of reasons. Often these proteins, whether of viral or cellular origin, themselves have additional functions. Examples are the NS1 protein of influenza virus, which regulates pre-mRNA splicing and nuclear export of poly(A)$^+$-RNA,[215] the cellular TAR RNA-binding protein, which augments expression of genes transcribed from retroviral long terminal repeats,[136,137] and the La antigen, which has been ascribed roles in both transcriptional and translational control. This last protein may act as a PKR inhibitor by virtue of a dsRNA unwinding activity[216] (M. James, G. Pruijn, and M.J. Clemens, unpublished observations). It is not clear to what extent the activity of these various inhibitors toward PKR is a secondary consequence of their other functions in cells. This makes it difficult to determine if these proteins are physiologically relevant regulators of the kinase *in vivo*.

Two other examples of proteins that are antagonistic to PKR have been described. One is the cellular protein p67, which protects eIF-2α from being phosphorylated by PKR in cells by binding to the substrate rather than the enzyme.[217] The other is the herpes simplex gene product $\gamma_1$34.5, which has recently been reported to enhance the activity of protein phosphatase 1α against phosphorylated eIF-2α.[218] In both cases, one would expect the effects of these proteins to be specific to the regulation of the eIF-2/eIF-2B system by PKR, with no consequences for the other pathways involving alternative substrates.

FUTURE DIRECTIONS

There are several areas of research where we may expect important and exciting developments in the PKR field in the near

TABLE 5. CELLULAR AND VIRAL PROTEIN INHIBITORS OF PKR AND THEIR MODES OF ACTION[a]

Protein	*Mode of action*	*Physiologic role*	*References*
Cellular proteins			
p58	Binds directly to PKR	Regulation of PKR in stressed cells(?)	74, 76, 77, 191, 208
TAR-BP	Binds dsRNA; dimerizes with PKR	Unknown	114, 136, 137
La antigen	Binds and unwinds dsRNA (?)	Control of translation and transcription	216
p67	Binds to eIF-2	Blocks phosphorylation by PKR	217
dRF	Inhibits dsRNA and ATP binding	Possible role in cell differentiation	207
Viral proteins			
HSV-1 $\gamma_1$34.5	Enhances activity of protein phosphatase 1α	Reduces phosphorylation of eIF-2α in HSV-1-infected cells	218
Vaccinia E3L	Binds dsRNA; dimerizes with PKR	Inhibition of PKR in vaccinia-infected cells	51, 213
Vaccinia K3L	Acts as pseudosubstrate	Inhibition of PKR in vaccinia-infected cells	212–214
Reovirus σ_3	Binds dsRNA	Inhibition of PKR in reovirus-infected cells	52
Influenza NS1	Binds dsRNA	Inhibition of PKR in influenza-infected cells	50, 215
HIV-1 Tat	Binds to and is phosphorylated by PKR *in vitro*; downregulates PKR *in vivo*	Reduces phosphorylation of eIF-2α in HIV-1-infected cells	154, 155, 224

[a]Dominant negative mutants of PKR itself are not included in this list.

future. One critical question is whether, in nonerythroid mammalian cells, PKR is unique in its ability to regulate the phosphorylation of eIF-2α or other substrates. For example, is PKR both necessary and sufficient for the control of the initiation of protein synthesis by such conditions as growth factor deprivation, amino acid starvation, heat shock, and other stresses that are known to cause the phosphorylation of eIF-2α? Related to this issue is the question of the mechanism(s) by which the expression of PKR inhibitors (including dominant negative mutants) causes phenotypic changes in cells and can result in tumorigenesis. Are these effects all due to the inhibition of endogenous PKR, or can other pathways be affected (particularly by those agents that act as competitive dsRNA-binding proteins[143])? What might be the roles of other proteins that appear to be immunologically related to PKR, such as those recently identified in Meurs' laboratory?[219,220] The use of murine embryonic fibroblasts derived from PKR knockout mice[150,151] will be valuable in answering several of these questions.

Another area where there are several interesting issues to be solved concerns the significance of PKR for the regulation of apoptosis. We can expect to see many developments in our knowledge of the mechanisms by which the kinase functions in this pathway, including the relationship of PKR to the actions of the *bcl*-2 gene family and the ICE-like proteases.[221] The regulation of apoptosis is especially relevant to the question of whether PKR can genuinely be regarded as a tumor-suppressor protein. If the latter can be established with certainty, it will also be important to search for deletions, translocations, or point mutations in the genes for PKR or its regulatory effectors in a variety of human tumors. Characterization of mutations associated with disease, particularly if they can be correlated with the functions of specific domains or individual autophosphorylation sites in the kinase, would potentially open the way to the development of clinically useful reagents for the control of PKR activity in target cells.

Finally, it will be of interest to learn how widely in the living kingdom the PKR pathway occurs. Recent evidence clearly points to a role for a PKR-like activity in plants, at least as far as resistance to virus infection is concerned.[18,19] Even in an organism as distantly related to mammals as the yeast *Saccharomyces cerevisiase*, PKR can functionally replace another eIF-2α kinase, the nutritionally regulated enzyme GCN2,[222] and PKR can also regulate the growth of yeast.[63] Thus, it would seem likely that PKR-like activities (whether regulated by dsRNA or by other mechanisms) could prove to be important in many organisms, with the potential for a myriad of practical applications in the future.

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Address reprint requests to:
Dr. Michael J. Clemens
Division of Biochemistry
Department of Cellular and Molecular Sciences
St. George's Hospital Medical School
Cranmer Terrace
London SW17 ORE
United Kingdom

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